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VERIFICATION OF TRANSLATION

Patent Application No. 204464/92

I, Hidejiro TANIGAWA of 9-3-1906, Hikarigaoka 3-chome, Nerima-ku, Tokyo 179-0072, Japan, am the translator of the documents attached and I state that the following is a true translation to the best of my knowledge and belief of Japanese Patent Application No. 204464/92.

DATED this 12th day of February, 2003

Signature of translator Hidejiro Tanigawa

Hidejiro TANIGAWA

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[TITLE OF THE INVENTION] Method for Transforming Monocotyledons

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[TITLE OF DOCUMENT] SPECIFICATION

[TITLE OF THE INVENTION] Method for Transforming Monocotyledons

[CLAIMS]

[CLAIM 1] A method for transforming a monocotyledon comprising transforming a cultured tissue during dedifferentiation process or a dedifferentiated cultured tissue of said monocotyledon with a bacterium belonging to genus Agrobacterium containing a desired gene.

[CLAIM 2] The method according to claim 1, wherein said monocotyledon is rice.

[CLAIM 3] The method according to claim 1 or 2, wherein said bacterium belonging to genus Agrobacterium has a plasmid containing a DNA fragment originated from the virulence region of a Ti plasmid pTiBo542 of Agrobacterium tumefaciens.

[CLAIM 4] The method according to claim 3, wherein said plasmid is pTOK162 or a derivative thereof.

[CLAIM 5] The method according to any one of claims 1 - 4, wherein said bacterium belonging to genus Agrobacterium is Agrobacterium tumefaciens.

[CLAIM 6] The method according to any one of claims 1 - 5, wherein cell population of said bacterium belonging to genus Agrobacterium used for transformation is 10^7 - 10^{10} cells/ml.

[CLAIM 7] The method according to any one of claims 1 - 6, wherein said cultured tissue is a cultured tissue

which has been placed on a callus induction medium for not less than 2 days and which is during callus formation process, or a callus.

[CLAIM 8] The method according to any one of claims 1 - 6, wherein said cultured tissue has an ability to regenerate a normal plant.

[DETAILED DESCRIPTION OF THE INVENTION]

[0001]

[INDUSTRIAL FIELD]

The present invention relates to a method for transforming monocotyledons.

[0002]

[PRIOR ART]

Conventional methods for transforming monocotyledons include electroporation method, polyethylene glycol method (PEG method), particle gun method and so on.

[0003]

The electroporation method is a method in which protoplasts and the desired DNA are mixed, and holes are formed in the cell membranes by electric pulse so as to introduce the DNA into the cells, thereby transforming the cells. This method currently has the highest reproducibility of the conventional methods and various genes have been introduced into monocotyledons, especially into rice plants by this method (Toriyama K. et al., 1988; Bio/Technol. 6:1072-1074, Shimamoto K. et al., 1989; Nature 338:274-276, Rhodes C.A. et al., 1989;

Science 240:204-207). However, this method has the problems that 1) it can be applied only to the plant species for which the system for regenerating plants from protoplasts has been established, 2) since it takes several months to regenerate plants from the protoplasts, a long time is required to obtain transformants, and that 3) since the culture period is long, the frequency of emergence of mutants during the culture is high accordingly, so that the probability of obtaining normal transformants is decreased.

[0004]

The PEG method is a method in which the desired gene and protoplasts are mixed and the mixture is treated with PEG, thereby introducing the gene into the protoplasts. This method is different from the electroporation method in that PEG is used instead of the electric pulse. The efficiency of introducing the gene is thought to be somewhat lower than the electroporation method. Although there is a report that transformants were obtained by this method, this method is not widely used. Since protoplasts are used, this method has the same problems as in the electroporation method (Zhang W. et al., 1988; Theor. Appl. Genet. 76:835-840, Datta S.K. et al., 1990; Bio/Technol. 8:736-740).

[0005]

The particle gun method is a method in which the desired gene is attached to fine metal particles, and the

metal particles are shot into cells or tissues at a high speed, thereby carrying out the transformation. Thus, according to this principle, transformation may be performed on any tissues. Therefore, this method is effective for transforming the plant species for which the systems for regenerating plants from protoplasts have not been established. The efficiency of transformation varies depending on the selection after the gene was shot. There is no data which compare the efficiency of this method with that of the electroporation method (Gordon-Kamm W.J. et al., 1990; Plant Cell 2:603-618, Fromm M.E. et al., 1990; Bio/Technol. 8:833-839, Christou P. et al., 1991; Bio/Technol. 9:957-962).

[0006]

Other methods include 1) culturing seeds or embryos with DNA (Topfer R. et al., 1989; Plant Cell 1:133-139, Ledoux L. et al., 1974 Nature 249:17-21); 2) treatment of pollen tube (Luo and Wu 1988; Plant Mol. Biol. Rep. 6:165-), 3) liposome method (Caboche M. 1990; Physiol. Plant. 79:173-176, Gad A.E. et al., 1990:177-183) and 4) microinjection method (Neuhaus G. et al., 1987; Theor. Appl. Genet. 75:30-36). However, these methods have problems in the efficiency of transformation, reproducibility or applicability, so that these methods are not commonly used.

[0007]

On the other hand, a method for introducing a gene

using the Ti plasmid of bacteria belonging to genus Agrobacterium as a vector is widely used for transforming dicotyledons such as tobacco, petunia, rape and the like. However, it is said that the hosts of the bacteria belonging to genus Agrobacterium are restricted to dicotyledons and that monocotyledons are not parasitized by Agrobacterium (De Cleene M. 1976; Bot. Rev. 42:389-466).

[0008]

As for transformation of monocotyledons by Agrobacterium, although transformation of asparagus (Bytebier B. et al., 1987: Proc. Natl. Acad. Sci. USA, 84:5345-5349) and of Dioscorea bulbifera (Schafew et al., 1987; Nature 327:529-532) has been reported, it is said that this method cannot be applied to other monocotyledons, especially to the plants belonging to family Gramineae (Potrykus I. 1990; Bio/Technol. 8:535-543).

[0009]

Grimsley et al. (1987: Nature 325:177-179) reported that T-DNA of Agrobacterium in which DNA of maize streak virus was inserted was inoculated to the apical meristem of maize plants and infection of the plants by maize streak virus was confirmed. Since the infected symptoms are not observed when merely the DNA of maize streak virus is inoculated, they interpreted the above-mentioned result as a piece of evidence showing that Agrobacterium

can introduce DNA into maize. However, since it is possible that a virus replicates even if it is not incorporated into the nucleus genome, the result does not show that the T-DNA was incorporated into the nucleus. They subsequently reported that the infection efficiency is the highest when the virus is inoculated to the apical meristem in the shoot apex of the maize (Grimsley et al., 1988: Bio/Technol. 6:185-189), and that virC gene in the plasmid of Agrobacterium is indispensable to the infection (Grimsley et al., Mol. Gen. Genet. 217:309-316).

[0010]

Gould J. et al. (1991; Plant Physiol. 95:426-434) inoculated super-virulent Agrobacterium EHA1 having a kanamycin-resistant gene and a GUS gene to shoot apices of maize after injuring the shoot apices with a needle, and selected the shoot apex based on the resistance to kanamycin. As a result, plants having resistance to kanamycin were obtained. They confirmed by Southern blotting analysis that some of the seeds of the subsequent generation of the selected plants had the introduced gene (chimera phenomenon).

[0011]

Mooney P.A. et al., (1991; Plant Cell, Tissue, Organ Culture 25:209-218) tried to introduce kanamycin-resistant gene into embryos of wheat using Agrobacterium. The embryos were treated with an enzyme to injure the

cell walls, and then Agrobacterium was inoculated. Among the treated calli, although very small number of calli which were assumed to be transformants grew, plants could not be regenerated from these calli. The existence of the kanamycin-resistant gene was checked by Southern blotting analysis. As a result, in all of the resistant calli, change in structure of the introduced gene was observed.

[0012]

Raineri et al. (1990; Bio/Technol. 8:33-38) inoculated super-virulent Agrobacterium A281 (pTiBo542) to 8 varieties of rice after injuring the scutella of the rice plants. As a result, growth of tumor-like tissues was observed in two varieties, Nipponbare and Fujisaka 5. Further, an Agrobacterium containing a plasmid having a T-DNA from which a hormone-synthesizing gene was removed and instead, a kanamycin-resistant gene and GUS gene were inserted therein was inoculated to embryos of rice. As a result, growth of kanamycin-resistant calli was observed. Although the expression of GUS gene was observed in these resistant calli, transformed plants could not be obtained from the calli. They interpreted these results as that the T-DNA was introduced into rice cells.

[0013]

Thus, although the experimental results which suggest that introduction of genes into the plants belonging to family Gramineae such as rice, maize and

wheat can be attained by using Agrobacterium have been reported, fully convincing results have not been obtained about the reproducibility, introduction efficiency and about the confirmation of the introduction of the gene (Potrykus I. 1990; Bio/Technol. 8:535-543).

[0014]

[PROBLEMS WHICH THE INVENTION TRIES TO SOLVE]

As mentioned above, introduction of genes into the plants belonging to family Gramineae is now mainly carried out by the electroporation method. However, with this method, since protoplasts are used, a long time and much labor are required to obtain regenerated plants. Further, there is a danger that mutants may emerge at a high frequency due to the long culturing period. Still further, this method cannot be applied to the plants such as maize for which the system for regenerating plants from protoplasts has not been established. In view of this, as mentioned above, as for maize, it has been tried to use the apical meristem. However, the operation for isolating the apical meristem requires much labor and it is not easy to prepare apical meristem in a large amount.

[0015]

Accordingly, an objective of the present invention is to provide a method for transforming monocotyledons, with which the time required for obtaining regenerated plants from the time of transformation is shorter than that in the conventional methods, which can be generally

applied even to the plants for which the systems for regenerating plants from protoplasts have not yet been established, and with which the preparation of the materials to be used is easy.

[0016]

[MEANS FOR SOLVING THE PROBLEMS]

The present inventors intensively studied the influences of the monocotyledonous plant tissues treated with Agrobacterium, treatment conditions of Agrobacterium, constitution of the binary vector and the like on the introduction efficiency of the genes to discover that cultured tissues of monocotyledons can be transformed by using Agrobacterium with drastically high efficiency and reproducibility and that by employing this method, the above-mentioned object can be attained, thereby completing the present invention.

[0017]

That is, the present invention provides a method for transforming a monocotyledon comprising transforming a cultured tissue during dedifferentiation process or a dedifferentiated cultured tissue of said monocotyledon with a bacterium belonging to genus Agrobacterium containing a desired gene.

[0018]

Detailed explanation of the invention is as follows.

[0019]

The monocotyledons which may be transformed by the

method according to the present invention are not restricted and the method according to the present invention may be applied to any monocotyledons such as rice, maize, barley, wheat, asparagus and the like.

[0020]

The cultured tissue employed in the present invention may be originated from any part of the plant. For example, cultured tissues originated from scutellum, shoot apex, root, pollen, and anther can be employed. As the cultured tissue employed in the present invention, it is preferred to employ a callus. A callus for each plant can be formed according to the known methods. It should be noted that the cultured tissue employed in the method of the present invention is not necessarily a callus, but suspension cells may also be employed.

[0021]

As the Agrobacterium used for the transformation, Agrobacterium which are employed for the transformation of dicotyledons can be employed. Many of these Agrobacterium contain a vector having a DNA region originated from the virulence region (vir region) of Ti plasmid originated from Agrobacterium tumefaciens. The gene encoding a character which is desired to be given to the plant is inserted in this vector, or exists in a separate plasmid and inserted into the Ti plasmid in vivo by homologous recombination or the like. The present inventors previously developed a vector containing a DNA

region originated from the virulence region (vir region) of Ti plasmid pTiBo542 (Jin S. et al., 1987: J. Bacteriol. 169:4417-4425) contained in a super-virulent Agrobacterium tumefaciens A281 exhibiting extremely high transformation efficiency (Hood E.E. et al., 1984; Bio/Technol. 2:702-709, Hood E.E. et al., 1986; J. Bacteriol. 168:1283-1290, Komari T. et al., 1986; J. Bacteriol. 166:88-94, Jin S. et al., 1987; J. Bacteriol. 169:4417-4425, Komari T. 1989; Plant Science 60:223-229 ATCC37394) (Japanese Laid-open Patent Application (Kokai) No. 4-222527. In this specification, this vector is also called "super binary vector"). Such a super binary vector may preferably be employed in the present invention.

[0022]

An example of such a super binary vector is pTOK162. The structure is shown in Fig. 1. This plasmid comprises a plasmid called pTOK154 which can replicate in both Escherichia coli and in Agrobacterium tumefaciens (pTOK154 is a plasmid containing T region, which was constructed by the method described below from a known plasmid pGA472 derived from the Ti plasmid and a known plasmid having a wide host spectrum called pVCK101), into which a Kpn I fragment (containing virB, virG and virC genes) with a size of 15.2 kb originated from the virulence region of pTiBo542 is inserted, the Kpn I fragment having been cloned. In pTOK154, between two

border sequences of the T region, a kanamycin-resistant gene is inserted as a gene to be introduced into a monocotyledon. This is an example wherein the gene desired to be introduced into the monocotyledon is arranged in a plasmid having the cloned DNA fragment originated from the virulence region of pTiBo542.

[0023]

The gene which is desired to be incorporated into the monocotyledon may be inserted into a restriction site in the T region of the above-described plasmid according to a general method, and the desired recombinant plasmid may be selected depending on an appropriate selection marker such as drug resistance and the like which the plasmid has. However, if the vector, like pTOK162 shown in Fig. 1, is large and has a number of restriction sites, it is not always easy to insert the desired DNA in the T region of the vector. In such a case, the desired DNA can be inserted in the T region of pTOK162 by utilizing the in vivo homologous recombination (Herrera-Esterella L. et al, 1983; EMBO J. 2:987-995, Horch R. H. et al. Science 1984; 223:496-498) in the cells of Agrobacterium tumefaciens. That is, pTOK162 is preliminarily introduced into Agrobacterium tumefaciens and the plasmid pBR322 (or a similar plasmid) containing the desired gene is further introduced into Agrobacterium tumefaciens. Since pTOK162 has a region homologous with a region of pBR322, the pBR322 derivative containing the

desired gene is inserted into pTOK162 by the genetic recombination via the homologous regions. Unlike pTOK162, pBR322 cannot replicate by itself in Agrobacterium tumefaciens. Therefore, pBR322 can only be alive in Agrobacterium tumefaciens in the inserted form in pTOK162 (the recombined pTOK162 and pBR322 is hereinafter designated "pTOK162::pBR322 derivative"). By selecting the transformants based on the selection markers (such as drug resistance) specific to each of pTOK162 and pBR322 derivative, Agrobacterium tumefaciens transformants containing pTOK162::pBR322 derivative may be obtained. The present inventors made a study by introducing various plasmids into Agrobacterium tumefaciens containing pTOK162, to discover that as the selection marker of the pBR322 derivative, spectinomycin-resistant gene (SP) originated from transposon Tn7 (De Greve H.H. et al., 1981; Plasmid 6:235-248) is suited. Thus, in cases where the desired gene has already been cloned into pBR322, by inserting SP gene into the plasmid, the desired DNA can be inserted in the T region of pTOK162 by homologous recombination in vivo in Agrobacterium tumefaciens. Alternatively, a plasmid containing the DNA from pBR322 and SP gene is first provided, and the desired gene may be inserted into this plasmid. In this case, by utilizing the border sequences of the T region, it is possible to arrange the kanamycin-resistant gene and the desired gene in separate T regions

in pTOK162. When plants are transformed using the resistance to kanamycin as a marker, there is a substantial probability that both T regions are introduced, the introduction of the desired gene can be sufficiently attained. Further, in this case, since both T regions may be inserted into different chromosomes, it may be possible to subsequently segregate the desired gene from the kanamycin-resistant gene.

[0024]

As the host bacterium belonging to genus Agrobacterium, Agrobacterium tumefaciens may preferably be employed, although not restricted.

[0025]

Introduction of a plasmid into the bacterium belonging to genus Agrobacterium such as Agrobacterium tumefaciens can be carried out by a conventional method such as triple cross method of bacteria (Ditta G. et al., 1980; Proc. Natl. Acad. Sci. USA 77:7347-7351).

[0026]

Since the Agrobacterium prepared as mentioned above has a highly virulent DNA originated from pTOK162, transformation of monocotyledons can be attained with a high efficiency.

[0027]

It should be noted that in the method of the present invention, although the gene which is desired to be introduced into the monocotyledon is arranged between

border sequences of the T region as in the prior art, the desired gene may be arranged in the Ti plasmid or in another plasmid in the Agrobacterium.

[0028]

The transformation of the cultured tissue of a monocotyledon by the Agrobacterium may be carried out by merely contacting the cultured tissue with the Agrobacterium. For example, a cell suspension of the Agrobacterium having a population density of 10^7 -10^{10} cells/ml is prepared and the cultured tissue is immersed in this suspension for 3 - 10 minutes. The resulting culture tissue is then cultured on a solid medium for several days together with the Agrobacterium. Alternatively, transformation may be carried out by adding the Agrobacterium to the culture medium of the cultured tissue and continuously culturing the cultured tissue together with the Agrobacterium.

[0029]

The transformed cultured tissue is then cultured by a known method. A plant having a desired gene introduced by transformation is thus regenerated.

[0030]

By the present invention, it was first attained to introduce foreign genes to monocotyledons such as plants belonging to family Gramineae including rice, maize, wheat, barley and the like with good reproducibility. Although methods for transforming monocotyledons using

Agrobacterium are known, they are not established methods as mentioned above. In contrast, according to the present invention, by inoculating Agrobacterium to cultured tissues which have not been employed in the conventional methods, genes can be very easily introduced. In the present invention, since a cultured tissue such as a callus which can be easily prepared is used, the sample materials can be obtained more easily than the conventional method employing the apical meristem. Further, since cultured cells are transformed, the time required for regenerating plants is shorter than in cases where protoplasts are transformed, so that the frequency of mutation is decreased. Further, by employing a super binary vector, it was first attained to introduce genes with high efficiency into some varieties of rice which are difficult to culture. Still further, as will be described in the examples below, by employing an appropriate selection method after inoculation, the chimera phenomenon in which the desired gene is introduced chimerally can be decreased.

[0031]

[EXAMPLES]

The present invention will now be described by way of examples thereof. It should be noted, however, that the present invention is not restricted to the examples.

[0032]

(1) Preparation of Sample Cultured Tissues

(i) Variety of Rice

Varieties Asanohikari, Tsukinohikari and Koshihikari, which are varieties of japonica rice were selected as samples.

[0033]

(ii) Scutellum and Scutellum Callus

Mature seeds of rice were sterilized by being immersed in 70% ethanol for 1 minute and then in 1% sodium hypochlorite solution for 30 minutes. The seeds were then placed on 2N6 solid medium (inorganic salts and vitamins of N6 (Chu C.C., 1978; Proc. Symp. Plant Tissue Culture, Science Press Peking, pp.43-50), 1g/l of casamino acid, 2 mg/l of 2,4-D, 30 g/l of sucrose, 2 g/l of Gelrite). Scutella were removed from the seeds on Day 4 from the beginning of the culture and used as "scutellum" samples. On the other hand, after culturing the mature seeds for about 3 weeks, the formed calli originated from scutella were transferred to 2N6 medium and cultured therein for 4 days. The resulting calli were used as "scutellum callus" samples.

[0034]

(ii) Shoot Apex Tissue

Mature seeds of rice were sterilized by the above-described method and were placed on 1/2 N6 solid medium (half strength of major inorganic salts and minor salts of N6, vitamins of N6, 1 g/l of casamino acid, 20 g/l of sucrose and 2 g/l of Gelrite). From seedlings on Day 3

after germination , tissues of 2 - 3 mm length containing apex dividing tissues were cut out and used as samples.

[0035]

(iv) Root Segment and Root Callus

From the seedlings obtained by the method descried in (iii), tip portions of 5 - 10 mm length of the seed roots were cut out and used as "root segment" samples. On the other hand, these radicles were cultured on 2N6 solid medium for about 2 weeks to obtain calli, and these calli were used as "root callus" samples.

[0036]

(v) Suspended Cultured Cells

The calli originated from scutella were transferred to AA liquid medium (major inorganic salts of AA, amino acids of AA and vitamins of AA (Toriyama and Hinata 1985; Plant Science 41:179-183), MS minor salts (Murashige and Skoog 1962; Physiol. Plant. 15:473-497), 0.5 g/l of casamino acid, 1 mg/l of 2,4-D, 0.2 mg/l of kinetin, 0.1 mg/l of gibberellin and 20 g/l of sucrose) and the cells were cultured therein at 25^{o}C in the dark under shaking of 120 rpm to obtain suspended cultured cells. The medium was replaced with fresh medium every week.

[0037]

(2) Ti Plasmid (Binary Vector)

Hygromycin resistant gene (HPT) and GUS gene were inserted in the T-DNA region of Ti plasmid to obtain the following plasmids:

[0038]

(i) pIG121 Hm:

A plasmid in which the GUS gene containing the first intron of the catalase gene of caster bean and a hygromycin-resistant gene were ligated (Nakamura et al., 1991; Plant Biotechnology II (Extra Issue of GENDAI KAGAKU, pp.123-132), presented from Dr. Nakamura of Nagoya University).

[0039]

(ii) pTOK232

1. Insertion of Intron GUS and Hygromycin-resistant Genes to Intermediate Vector pTOK229

The Cla I fragment (2.5 kb) of the spectinomycin-resistant gene originated from Tn7 were treated with Klenow fragment to blunt the ends. The resulting fragment was inserted in Sma I site of pUC19 to obtain a plasmid pTOK107 (5.2 kb) having ampicillin-registant and spectinomycin-resistant genes. The obtained pTOK107 was treated with Eco RI and Hind III and the obtained 2.5 kb fragment containing the spectinomysin-resistant gene was ligated to a Eco RI - Hind III fragment (2.7 kb) of pGA482 to obtain pTOK170 (5.2 kb) containing the spectinomycin-resistant gene and has Hind III site and Hpa I site.

[0040]

A vector pIG221 in which the first intron of catalase of caster bean and GUS gene are ligated to 85S

promoter (Ohta S. et al., 1990, presented by Dr. Nakamura of Nagoya University) was digested with Eco RI and the resultant was treated with Klenow fragment to blunt the ends. To the resultant, a Hind III linker (pCAAGCTTG, code 4660P commercially available from TAKARA SHUZO). A fragment containing 35S promoter and intron GUS was cut out by digesting the resulting vector with Hind III, and the fragment was inserted into the Hind III site of a plasmid pGL2 (J. Paszkowski, obtained from Friedrich Mieocher Institute) containing a hygromycin-resistant gene ligated to 35S promoter, to obtain pGL2-IG (7.6 kb). The above-mentioned plasmid pGL2 was obtained by inserting a hygromycin-resistant gene (Gritz L. and Davis J. 1983; Gene 25: 179 - 188) into pDH51 (Pietrazak et al., 1986; Nucleic Acids Research 14: 5857 - 5868). The fragment obtained by treating pTOK170 with Hpa I was ligated to a Pvu II fragment (5.2 kb) of pGL2-IG to obtain pTOK229 (10.1 kb).

[0041]

2) Insertion into Super Binary Vector pTOK162

Insertion of the desired genes (hygromycin-resistant gene and intron GUS gene) into a super binary vector pTOK162 obtained by inserting virB, virC and virG genes of strongly virulent Agrobacterium tumefaciens A281 into the binary vector was carried out by homologus recombination. That is, since both vectors contain a region originated from an E. coli plasmid pBR322, in the

bacterial cells selected by resistances to spectinomycin and kanamycin, only the plasmid generated by recombination of the both plasmids are contained. The plasmid obtained by the fact that the hygromycin-resistant gene and the intron GUS gene were inserted into the super binary vector is called pTOK232 (see Fig. 1).

[0042]

(3) Host Agrobacterium tumefaciens

Strains LBA4404 and EHA101 in which T-DNA regions were deleted were used as the host bacteria. Strain LBA4404 has a helper plasmid PAL4404 (having a complete vir region), and is available from American Type Culture Collection (ATCC 37349). Strain EHA101 has a helper plasmid having the vir region originated from a strongly virulent Agrobacterium tumefaciens A281, and is available from Hood E.E. et al., 1986.

[0043]

The various binary vectors described in (2) were introduced into these two strains of Agrobacterium tumefaciens, and the strains described in the following were used for introducing the genes. The plasmids were introduced into the Agrobacterium strains by triple cross (Ditta G. et al., 1980; Proc. Natl. Acad. Sci. USA 77: 7347-7351).

LBA4404(pTOK232)

LBA4404(pIG121Hm)

EHA101(pIG121Hm)

[0044]

(4) Preparation of Suspension of Agrobacterium tumefaciens

Colonies obtained by culturing the Agrobacterium strains on AB medium (Drlica K.A. and Kado C.I. 1974; Proc. Natl. Acad. Sci. USA 71:3677-3681) containing hygromycin (50 μg/ml) and kanamycin (50 μg/ml) for 3 - 10 days were collected with a platinum loop and suspended in modified AA medium (same as the composition of the above-described AA medium except that concentrations of sucrose and glucose were changed to 0.2 M and 0.2 M, respectively, and that 100 μM of acetosyringone was added, pH 5.2). The cell population was adjusted to 3 x 10^9 - 5 x 10^9 cells/ml and the suspensions were used for inoculation.

[0045]

(5) Inoculation Conditions

The sample tissues were washed with sterilized water and immersed in the above-described suspensions of Agrobacterium strains, respectively, for 3 - 10 minutes. Thereafter, the shoot apex samples were placed on N6S3 solid medium (1/2 N6 major inorganic salts, N6 minor salts, N6 vitamins, Chu C.C., 1978, AA amino acids (Toriyama and Hinata 1985), 1 g/l of casamino acid, 0.2 mg/l of NAA, 1.0 mg/l of kinetin and 3 g/l of Gelrite) containing 100 μM of acetosyringone, 10 g/l of glucose and 20 g/l of sucrose. The other tissue samples such as

scutellum callus samples were cultured on 2N6 solid medium containing acetosyringone, glucose and sucrose in the same concentrations as mentioned above. The both culture was carried out at 25°C in the dark for 2 - 5 days. The resulting inoculated tissues were then washed with sterilized water containing 250 mg/l of cefotaxime and then continued to be cultured on the respective solid media containing the same concentration of cefotaxime as mentioned above.

[0046]

(6) Method for Examining GUS Activity

Immediately after the above-mentioned culture with the Agrobacterium strains, the tissues were immersed in 0.1 M phosphate buffer (pH 6.8) containing 0.1% TRITON X-100 at 37°C for 1 hour. After washing off the Agrobacterium strains with phosphate buffer, phosphate buffer containing 0.1 mM of 5-bromo-4-chloro-3-indolyl-β-D-gluconic acid and 20% methanol was added to the tissues. After incubation at 37°C for 24 hours, the number of blue-colored tissues were counted under a microscope and the percentages thereof based on the number of samples are described. In the judgment of the GUS activities of the plants assumed to be transformants after the selection treatment, leaves were collected from the plants and GUS staining was performed in the same manner.

[0047]

(7) Selection of Transformed Cells and Tissues

(i) Shoot Apex

Shoot apices cultured with the Agrobacterium strains for 5 days were cultured on N6S3 medium containing 250 mg/l of cefotaxime for 2 weeks. The grown shoot apex tissues were transplanted to N6S3 medium containing 40 mg/l of hygromycin and selection of the transformants was carried out.

[0048]

(ii) Cultured Tissue (Scutellum Callus)

Tissues cultured with the Agrobacterium strains for 3 days were cultured on 2N6 medium containing 250 mg/l of cefotaxime for 1 week. Hygromycin-resistant cultured tissues were selected by culturing the cultured tissues on 2N6 medium containing 50 mg/l of hygromycin for 3 weeks (primary selection). The obtained resistant tissues were further cultured on N6-12 medium (N6 inorganic salts, N6 vitamins, 2 g/l of casamino acid, 0.2 mg/l of 2,4-D, 0.5 mg/l of 6BA, 5 mg/l of ABA, 30 g/l of sorbitol, 20 g/l of sucrose and 2 g/l of Gelrite) containing 50 mg/l of hygromycin for 2 - 3 weeks (secondary selection), and the calli grown on this medium were transferred to a plant regeneration medium N6S3 containing 0, 20 or 50 mg/l of hygromycin. In all of the media used after the culture with Agrobacterium strains, cefotaxime was added to 250 mg/l.

[0049]

(10) Differences in Efficiencies of Introducing Genes Depending on Sample Tissues (Expression of GUS after Culturing with Agrobacterium Strains)

In order to confirm that Agrobacterium can introduce genes into cells of monocotyledons, various tissues of the rice variety Tsukinohikari were treated with Agrobacterium tumefaciens EHA101 having a super-virulent vir region, into which the binary vector pIG121Hm (described above) containing the hygromycin-resistant gene and the GUS gene were introduced, and then the GUS activities were examined. The sample tissues were shoot apices, radicles, scutella, radicle calli, scutellum calli and suspended cultured cells. In cases where the tissues were not treated with the Agrobacterium strain, no tissues exhibited GUS activity indicated by blue color. On the other hand, in cases where the tissues were treated with Agrobacterium tumefaciens EHA101 (pIG121Hm), in all of the tissues except for radicles, expression of GUS was confirmed. The ratio of the number of the tissues showing blue color to the number of treated tissues was the highest in scutellum calli (Table 1). Further, the size of the tissues expressing GUS was also largest in scutellum calli. The tissues exhibited the second highest rate of introduction next to the scutellum calli were shoot apices. However, when the shoot apex tissues were selected by hygromycin-resistance, it was found that all of the tissues were

dead and no hygromycin-resistant tissue was obtained. The shoot apices have the apical meristem, and requires to have a gene introduced into the restricted region near the apical meristem, so that resistant tissues may grow after gene introduction. From the fact that many genes could be introduced into the shoot apices after culturing with Agrobacterium, but no resistant tissue was obtained from the tissues, the rate of gene introduction in the region near the apical meristem is assumed to be low. Therefore, scutellum calli or calli originated from other tissues are preferably employed as sample material for culture with Agrobacterium.

Table 1

Differences in Efficiency of Introduction of GUS Gene Depending on Sample Material (Variety:Tsukinohikari)

Sample Tissue	Number of GUS+ Tissues /Number of Sample Tissues(%)		Size of GUS-stained Portion Based on Treated Tissue
	Non-Treated Group	Treated Group	
Shoot Apex	0/ 30 (0)	109/157 (69)	+++
Root Segment	0/ 20 (0)	0/ 30 (0)	
Root Callus	0/ 30 (0)	24/115 (21)	+
Scutellum	0/ 50 (0)	8/ 89 (9)	+
Scutellum Callus	0/141 (0)	312/395 (79)	+++
Suspension Cells	0/232 (0)	61/247 (25)	++

+ : 1% or less, ++ : 1～10%, +++ : 10% or more

[0050]

It has been confirmed that the binary vector pIG121Hm used in this experiment does not express GUS gene in Agrobacterium cells because the intron of caster oil plant is inserted in the promoter of the GUS gene (Nakamura et al., 1991). Thus, from the results of the experiments described above in which the expression of the GUS gene after the culturing with Agrobacterium is used as an index, it was confirmed that genes can be introduced into rice cells by Agrobacterium.

[0051]

(9) Differences in Efficiencies of Introducing Genes Depending on Varieties of Rice (Expression of GUS after Culturing with Agrobacterium Strains)

There are large differences among varieties about the conditions for establishing cultured cells and for regenerating plants from the cultured cells (Mikami and Kinoshita 1988; Plant Cell Tissue Organ Cult. 12:311 - 314). It is said that Koshihikari is difficult to culture among the Japonica rices. On the other hand, Tsukinohikari employed in the preceding section is relatively easy to culture. When using the transformation method utilizing Agrobacterium, it is practically inconvenient if such differences among varieties exist. In order to clarify this point, the differences in the efficiencies of gene introduction between Koshihikari and Tsukinohikari which have

different easiness to culture were examined. The sample tissues employed were scutellum calli and the Agrobacterium tumefaciens strains employed were EHA101(pIG121Hm) and LBA4404(pIG121Hm).

[0052]

While GUS activity was observed in not less than 90% of calli of Tsukinohikari in each experiment, the GUS activity was observed in Koshihikari at lower rates (Table 3). Thus, in cases where EHA101(pIG121Hm) or LBA4404(pIG121Hm) is used, there is a difference in the introduction efficiency between the varieties.

Table 2

Differences in Rate of Introduction of GUS Gene Depending on Agrobacterium Strain and Rice Variety

		Number of GUS+ Tissues / Number of Treated Tissues (%)		
		Strain		
Variety	Experiment	LBA4404 (pIG121Hm)	EHA101 (pIG121Hm)	LBA4404 (pTOK232)
Tsukinohikari	1	67/70 (96)	78/87 (90)	64/66(97)
Tsukinohikari	2	72/86 (84)	68/73 (93)	82/82(100)
Koshihikari	1	46/135(34)	43/116(37)	124/131(95)
Koshihikari	2	28/107(26)	81/143(57)	102/103(99)

[0053]

(13) Differences in Efficiencies of Introducing Genes Depending on Agrobacterium Strains (Expression of GUS Gene after Culturing with Agrobacterium Strains)

EHA101(pIG121Hm) has a helper plasmid containing the vir region of super-virulent Agrobacterium tumefaciens A281. LBA4404(pIG121Hm) has an ordinary vir region. On the other hand, although the vir region of the helper plasmid in LBA4404(pTOK232) is ordinary, a gene which is a part of the vir region of the super-virulent Agrobacterium tumefaciens A281 is contained in the binary vector. This binary vector is originated from pTOK162 and made it possible to transform at a very high rate dicotyledonous species which are difficult to transform (Saito Y. et al., 1992; Theor. Appl. Genet. 83:679-683). Thus, there is a possibility that the transformation efficiency is largely influenced by the existence a super-virulent vir region or by the manner of existence thereof. Thus, using the above-described three Agrobacterium strains whose super-virulent vir regions are different, the efficiencies of introducing GUS gene were compared. The samples used were scutellum calli of Koshihikari and Tsukinohikari.

[0054]

Even with LBA4404(pIG121Hm) which does not have a super-virulent vir region, tissues exhibiting GUS activities were obtained in both varieties. However, in

Koshihikari, the rate was as low as about 30%. With EHA101(pIG121Hm) having the super-virulent vir region in the helper plasmid, the introduction efficiency in Koshihikari was somewhat higher. With LBA4404(pTOK232) having the super-virulent vir region in the binary vector, GUS activities were observed in not less than 95% tissues even with Koshihikari as with Tsukinohikari (Table 2). Further, as for the area of blue-colored regions in each tissue, the area was the largest with LBA4404(pTOK232), which indicates a high introduction efficiency.

[0055]

(11) Differences in Selection Efficiency Depending on Strains (Hygromycin-resistant Callus)

Using the above-mentioned 3 strains, the selection rates of hygromycin-resistant calli after culturing scutellum calli of Tsukinohikari and Koshihikari with the Agrobacterium strains were compared. As for the rate of emergence of the resistant calli, LBA4404(pTOK232) exhibited the highest rate. No differences about the rate of selection were observed between the varieties (Table 4). With the strains LBA4404(pIG121Hm) and EHA101(pIG121Hm), the rates of selection were low. Especially, with Koshihikari which is difficult to culture, the rate of emergence of hygromycin-resistant calli was as low as about 2%. Thus, it is thought that LBA4404(pTOK232) having a part of the super-virulent vir

gene in the binary vector is the best as the Agrobacterium strain used for transforming rice.

Table 3
Differences in Transformation efficiency Depending on Agrobacterium Strain (Scutellum Callus)

		Number of Hygromycin-resistant Callus / Number of Treated Callus (%)		
		Strain		
Variety	Experiment	LBA4404 (pIG121Hm)	EHA101 (pIG121Hm)	LBA4404 (pTOK232)
Tsukinohikari	1	91/338 (27)	139/301 (46)	169/305 (55)
Tsukinohikari	2	59/421 (14)	66/425 (16)	110/360 (31)
Tsukinohikari	3		10/521 (2)	174/644 (27)
Tsukinohikari	4		20/349 (6)	100/349 (29)
Koshihikari	1	6/269 (2)		65/283 (23)

[0056]

(12) Manner of Expressing GUS Gene in Hygromycin-resistant Transformants

The thus obtained resistant calli were subjected to secondary selection, and plants were regenerated from the selected resistant calli. A group in which hygromycin was not added to the N6S3 medium for regeneration was provided. In this group, a number of plants which did not exhibit GUS activity or which exhibited GUS activity chimerally emerged. However, in cases where hygromycin was added to the regeneration medium, the number of these plants largely decreased and the number of plants each of which exhibits GUS activity in the whole plant was increased (Table 5, Table 6 and Table 7). In cases where the tissue was not treated with Agrobacterium, no plants which exhibited resistance to hygromycin or GUS activity were obtained. Therefore, the plants each of which exhibited GUS activity in the whole plant, which was regenerated from the hygromycin-resistant callus, are considered as transformants.

Table 4

Expression of GUS Gene in Plants Regenerated from Hygromycin-resistant Calli (Variety:Asanohikari, Strain: EHA101(pIG121Hm)

Resistant Callus	Number of Regenerated Plants	Expression of GUS Gene		
		Stably Positive	Chimera	Negative
1	26	25	1	0
2	8	7	1	0

(Hygromycin was added to culture medium until regeneration of plants.)

[BRIEF DESCRIPTION OF THE DRAWINGS]

[Fig.1]

Fig.1 shows the structure of pTOK162 which is an example of the plasmid contained in Agrobacterium bacteria that may be employed in the method of the present invention, and shows the method for constructing a plasmid pTOK232 used in the examples of the present invention.

[DESCRIPTION OF REFERENCE SYMBOLS]

SP: spectinomycin-resistant gene

HPT: hygromycin-resistant gene

NPT: kanamycin-resistant gene

TC: tetracycline-resistant gene

IG: intron GUS gene

BR: right border sequence of T-DNA

BL: left border sequence of T-DNA

virB, virC, virG: vir regions originated from super-virulent Agrobacterium tumefaciens A281

ORI: replication origin of ColE1

COS: COS site of λ phage

K: restriction enzyme Kpn I site

H: restriction enzyme Hind III site

[TITLE OF DOCUMENT] ABSTRACT

[ABSTRACT]

[OBJECT] To provide a method for transforming monocotyledons, with which the time required for obtaining regenerated plants from the time of transformation is shorter than that in the conventional methods, which can be generally applied even to the plants for which the systems for regenerating plants from protoplasts have not yet been established, and with which the preparation of the materials to be used is easy.

[CONSTRUCTION] The invention provides a method for transforming a monocotyledon comprising transforming a cultured tissue during dedifferentiation process or a dedifferentiated cultured tissue of said monocotyledon with a bacterium belonging to genus Agrobacterium containing a desired gene.

[SELECTED FIGURE] Fig.1

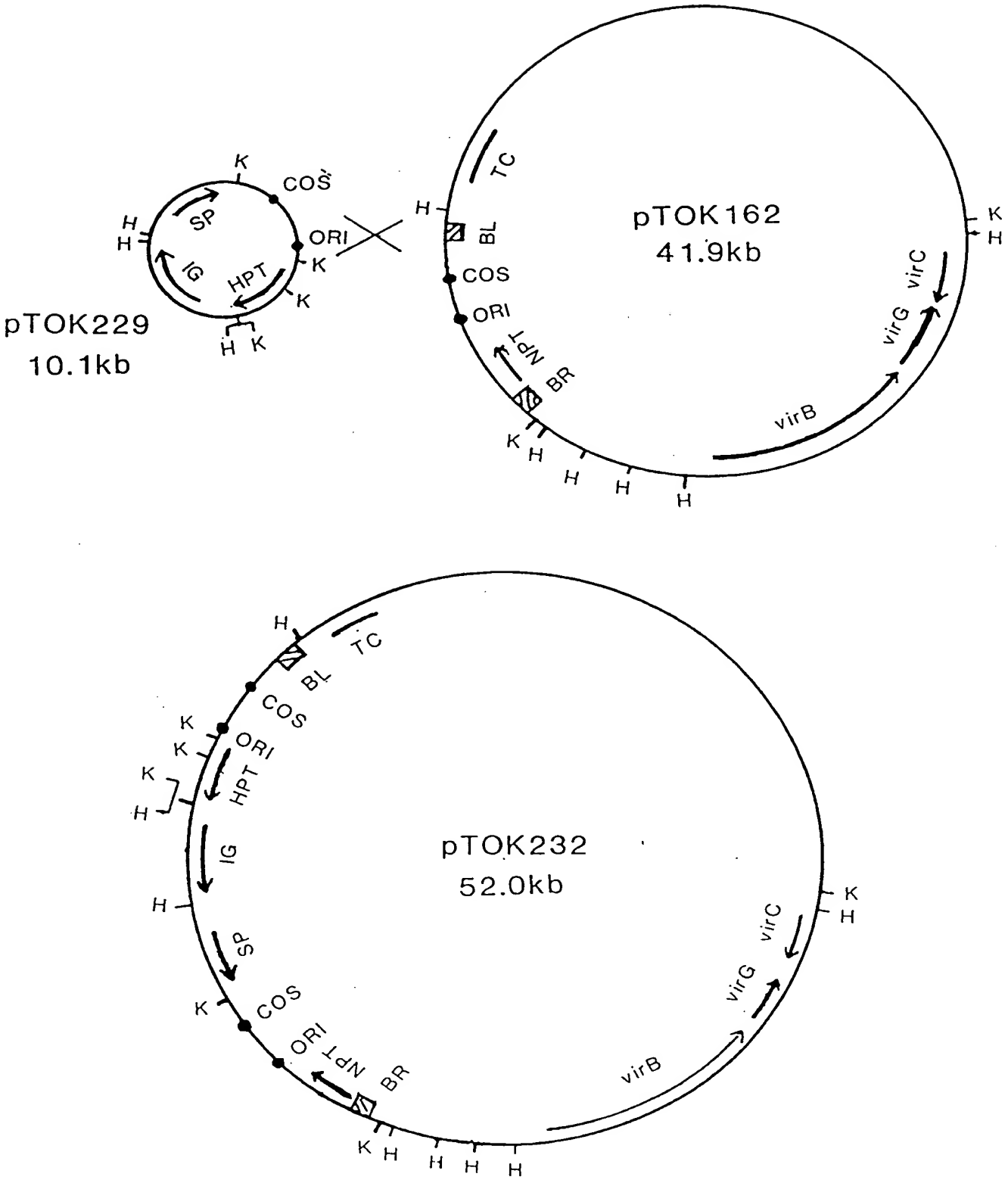
pTOK229
10.1kb
pTOK162
41.9kb
pTOK232
52.0kb
COS
ORI
SP
IG
HPT
BL
TC
BR
NPT
virB
virG
virC
K
H

FIG. 1